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Alteration of the erythrocyte membrane via enzymatic degradation of ankyrin (band 2.1): subcellular surgery characterized by EPR spectroscopy

Kenneth Hensley, Jennifer Postlewaite, Pamela Dobbs and D. Allan Butterfield

Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY (USA)

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A fraction of band 3 protein, the major transmembrane protein of erythrocyte membranes, is held to the cytoskeletal protein spectrin via noncovalent interactions with the protein ankyrin (band 2.1). In this study, trypsin was used under defined conditions to selectively proteolyze ankyrin and thereby destroy the band 3-ankyrin linkage on the cytoplasmic side of erythrocyte ghost membranes. Electron paramagnetic resonance (EPR) spectroscopy, in conjunction with selective spin labeling methods, was used to monitor conformational changes occurring in cytoskeletal proteins or cell-surface carbohydrates as a result of this treatment. Treatment of RBC ghosts with TPCK-trypsin for 5 s at 0°C caused an approx. 56% increase in the relevant EPR parameter of a maleimide spin label bound to spectrin ($P < 0.004$), indicative of increased segmental motion of the spin label and decreased protein–protein interactions. Analysis of the apparent rotational correlation time parameter τ of a spin label covalently and selectively bound to terminal sialic acid residues of glycophorin showed no significant effect from trypsin treatment. However, τ of spin label covalently and specifically bound to terminal galactose residues of cell-surface glycoconjugates of band 3 and other transmembrane glycoproteins significantly decreased with tryptic uncoupling of the ankyrin linkage ($P < 0.005$). These results suggest a marked conformational alteration in both cytoskeletal and transmembrane proteins as a result of uncoupling from ankyrin. Spermine (N,N'-bis(3-aminopropyl)tetramethylenediamine), a naturally occurring polyamine known to strengthen cytoskeletal protein-protein interactions (Wyse and Butterfield (1988) Biochim. Biophys. Acta 941, 141–149), was used to partially reverse the trypsin-induced cytoskeletal alterations. Addition of 2 mM spermine to ghosts previously treated with trypsin increased cytoskeletal protein-protein interactions as indicated by EPR ($P < 0.002$). SDS-PAGE was used to confirm the integrity of spectrin, band 3, and band 4.1 in all experiments. The results are discussed with reference to transmembrane signaling mechanisms and membrane-associated pathologies.

Introduction

The human erythrocyte (red blood cell, RBC) cytoskeleton consists of a protein meshwork which laminates the cytoplasmic face of the RBC membrane and is connected to transmembrane proteins via noncovalent interactions (Fig. 1). The principal component of the cytoskeleton is spectrin, which attaches to the membrane at two points. A junctional complex of actin and band 4.1 protein links the spectrin meshwork to the transmembrane protein glycophorin, and ankyrin (band 2.1 protein) connects spectrin to a fraction of band 3 anion transporter molecules [1–3,8]. A properly functioning cytoskeleton gives the RBC the durability to survive shear stress in the heart as well as flexibility to pass through ultrafine capillary beds. In addition to its mechanical function, the cytoskeleton subserves many biochemical processes. Several cytoskeletal proteins are known substrates for enzymes and regulatory proteins (e.g., protein kinases [1,2], glycolytic enzymes [2], calmodulin [1,10] and calcium-dependent proteinases [3,7]). Cytoskeletal-associated transmembrane proteins such as band 3 and glycophorin function as cell-surface receptors and signal transducers and as immunogenic targets in senescent or diseased cells [2,3,7,9,10].

Alteration of cytoskeletal components may affect cellular deformability and integrity [8,13,14], produce new cell-surface antigens [3,7], and disturb cellular physiology. Disruption of cytoskeletal-transmembrane linkages can be caused by accumulation of hemoglobin breakdown products [15], activity of endogenous proteinases [3,7], mutations in linkage proteins [16,17], or

Correspondence to: D.A. Butterfield, Department of Chemistry and Center of Membrane Sciences, University of Kentucky, Lexington, KY 40506-0055, USA.

other processes. Weakening of cytoskeletal protein–protein interactions has been associated with a host of conditions including hereditary spherocytosis (some forms of which may be caused by an ankyrin mutation [16]), generation of senescent cell antigen (an event possibly preceded by proteolysis of the band 3 cytoplasmic domain [3,7]), and Alzheimer's disease (AD) [22]. Our laboratory has recently shown that some therapeutic agents (tacrine, velnacrine) which improve mentation in AD victims, act to strengthen cytoskeletal linkages in RBCs and neocortical synaptosomes [11,12,19].

The band 3-ankyrin linkage merits particular study because of its crucial but poorly understood role in pathology and in regulation of cell physiology and generation of senescent cell antigen. A better understanding of the ankyrin-band 3 linkage could yield valuable insight into phenomena ranging from blood diseases to autoimmune disorders and degenerative conditions.

Experimental methods have recently been developed to selectively proteolyze ankyrin and thereby destroy the band 3-ankyrin linkage in otherwise normal cells [13,20]. Under rigorously controlled conditions it is reported that the proteinase trypsin will attack sensitive lysine and arginine residues of ankyrin while leaving other constituents of the RBC membrane unharmed [13,20]. The effect of ankyrin-band 3 uncoupling on the physical state of membrane cytoskeletal proteins and cell-surface carbohydrates has not been effectively characterized.

In this study, methods were employed to selectively attach nitroxide labels to thiol groups on spectrin, terminal sialic acid (NANA) residues on glycophorin oligosaccharides (70% of cell-surface sialic acid resides on glycophorin [8]), or terminal galactose residues on cell-surface glycoconjugates (including band 3) as previously described [22–24]. TPCK-trypsin was used to digest ankyrin, and physical alterations in labeled membrane components were characterized by EPR spectroscopy.

Materials and Methods

Chemicals

Nitroxide spin labels 2,2,6,6-tetramethyl-4-maleimidopiperidin-1-oxyl (MAL-6) and 2,2,6,6-tetramethyl-4-aminopiperidin-1-oxyl (tempamine) were obtained from either Sigma or Aldrich. $NaAsO_2$, $NaIO_4$, and $NaBH_3CN$ used in the labeling procedures were obtained from Sigma, as were the proteinase inhibitors phenylmethylsulfonyl fluoride (PMSF), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), and L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK). Galactose oxidase, type XIII TPCK-trypsin and type II-S soybean trypsin inhibitor were obtained from Sigma.

Blood was obtained from healthy human volunteers by venipuncture into heparinized tubes, and placed on ice. Processing of blood began within 30 min of collection. Whole blood was washed three times in phosphate buffered saline (5 mM phosphate/150 mM NaCl (pH 8.0)), pelleted by centrifugation at $600 \times g$ for 5 min. at 4°C, and the supernatant and buffy coat removed by aspiration. RBC membranes (ghosts) were isolated by osmotic lysis in hypotonic 5P8 (5 mM phosphate (pH 8.0)) at 4°C. Hemoglobin was removed by five consecutive washes in 5P8, followed each time by centrifugation at $27000 \times g$ for 10 min at 4°C.

Spin labeling of carbohydrates

Terminal galactose residues of cell-surface glycoconjugates (including band 3) or terminal sialic acid

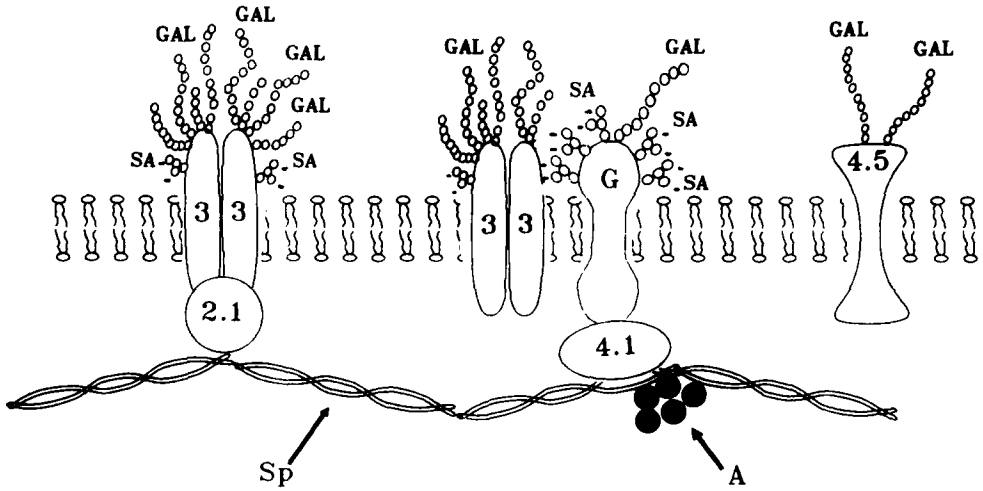

Fig. 1. Schematic diagram showing the orientation in the membrane of key molecules discussed in this study (note that only 1/6 of band 3 molecules are coupled to spectrin via band 2.1 [1]). 3, band 3; G, glycophorin; SA, sialic acid residues; GAL, galactose residues; 4.5, band 4.5; Sp, spectrin; 2.1, band 2.1 or ankyrin; A, actin.

residues of glycophorin in membrane ghosts were covalently and selectively spin labeled, as desired, by reductive amination procedures described previously [23,24]. In the case of galactose labeling, intact cells were activated by galactose oxidase to produce an aldehyde on C-6 terminal galactose and galactosamine residues, while ghosts were used in the sialic acid activation process with $NaIO_4$. Protein concentrations were assayed by the method of Lowry [26].

Spin labeling of cytoskeletal proteins

The labeling solution consisted of 51 μM MAL-6 in 5P8, well below the 1 mM level of SH-specific reagents required to disrupt the spectrin tetramer-dimer equilibrium [27]. Thiol groups of spectrin were labeled by incubating 1 ml ghosts (at 3 mg protein/ml ghosts) with 10 ml MAL-6 solution at 4°C for 14–16 h as previously described [22].

TPCK-trypsin treatment

Ghosts were washed twice in 5P8 and twice in 10 mM Tris (pH 7.4) to remove excess spin label and to prepare the membranes for trypsin treatment.

Ice-cold solutions of TPCK-trypsin and trypsin inhibitor were prepared fresh by dissolving these agents in 10 mM Tris (pH 7.4). Ghosts were assayed for total protein content and pipetted into 5 ml culture tubes suspended in an ice bath. Ice-cold TPCK-trypsin was introduced into ghost suspensions on ice to give a final concentration of 1 mg trypsin: 300 mg membrane protein. At specific time periods, the trypsin was deactivated by addition of at least 10-fold excess of trypsin inhibitor. Ghosts were washed once more in 5P8, assayed for protein content, and aliquots withdrawn for EPR analysis or SDS-PAGE.

Spermine treatment

Fresh spermine solutions (pH 8.0), were prepared at 8 mM by dissolving spermine in 5P8 buffer and titrating with a measured volume of 1.0 M HCl. Typically, a 100 ml solution of 8 mM spermine required 0.8–1.2 ml HCl to reach the desired pH. An ionic strength control solution was prepared by addition of a NaCl aliquot (0.5 M) to an appropriate volume of 5P8 [28]. Solutions so prepared were kept on ice until ready to use.

Several minutes prior to spectral acquisition, 1 volume of 8 mM spermine was added to 3 volumes of spin-labeled ghosts to yield a final spermine concentration of 2.0 mM and a final protein concentration of 2 mg/ml. Ionic strength controls were prepared by substituting the NaCl control solution for spermine. 'Ghost only' controls were prepared by substituting 5P8 for spermine or NaCl. Ghost suspensions thus treated were allowed to equilibrate to room temperature for 15 min prior to spectral acquisition.

EPR / SDS-PAGE

EPR spectroscopy was performed at a protein concentration of 2.0–2.5 mg/ml in a flat quartz aqueous sample cell, using a Bruker 300 ESP spectrometer equipped with computerized data acquisition and analysis capabilities (microwave frequency = 10 GHz, microwave power = 10 mW, modulation frequency = 100 kHz, modulation amplitude = 0.481 G, time constant = 1.28 ms). PAGE was performed according to the method of Fairbanks et al. [29], using a continuous buffer system and a 4.6% acrylamide gel, and protein bands were visualized by staining with Coomassie brilliant blue R-250.

Results

MAL-6 labeled spectrin / SDS-PAGE

The physical state of cytoskeletal proteins of erythrocyte membranes was monitored by EPR using the protein specific spin label MAL-6 [22]. By use of selective isolation experiments and studies employing antibodies to MAL-6 [4,22], it is reported that up to 90% of the spin label intensity of MAL-6 is found on spectrin, the major cytoskeletal protein. The relevant EPR parameter measured is the ratio of the spectral amplitude of the $M_I = +1$ low-field weakly immobilized line (W) and that of the $M_I = +1$ low-field strongly immobilized line (S), which is referred to as the W/S ratio (Fig. 2). Changes in the W/S ratio are known to be strong indicators of perturbations in the normal interactions of cytoskeletal elements [15,22,25,28]. Previous studies have shown that decreased cytoskeletal protein–protein interactions caused by hemin [15], polyphosphates [5] or increased dimeric spectrin content [25] 'loosen' the conformation

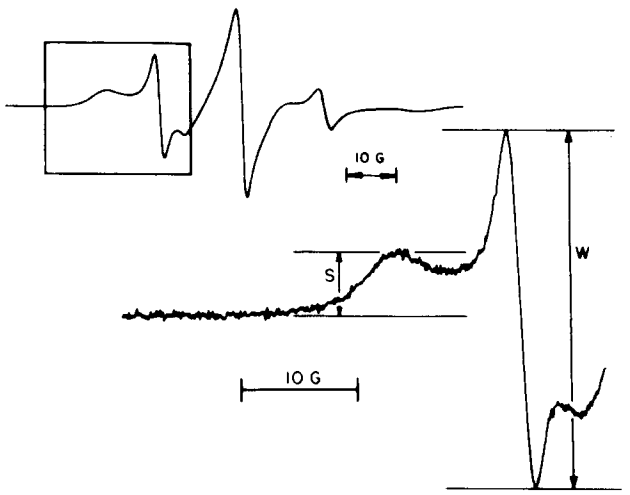

Fig. 2. Typical EPR spectrum of MAL-6 covalently attached to erythrocyte membrane proteins, showing strongly (S) and weakly (W) immobilized components of the $M_I = +1$ low-field resonance line (box).

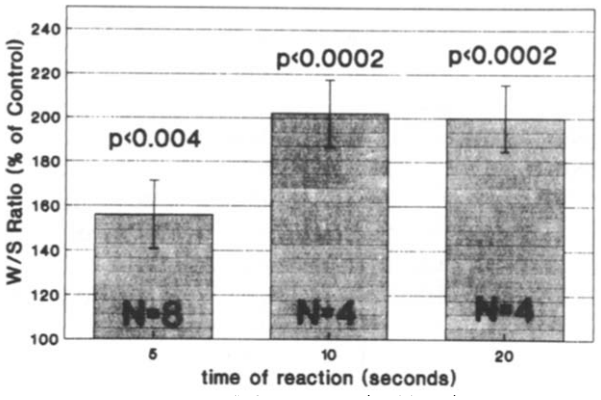

Fig. 3. EPR analysis of the W/S ratio as a function of TPCK-trypsin treatment. P-values computed by one-tailed Student's t-test. Error bars indicate S.E.

of spectrin and decrease steric hindrance to spin label motion resulting in an increase in the W/S ratio of MAL-6.

Fig. 3 summarizes the effect of trypsin to decrease cytoskeletal protein–protein interactions as judged by EPR. A significant increase was seen in the W/S ratio of MAL-6 labeled ghosts following as little as 5 s TPCK-trypsin incubation at 0°C (mean increase over controls $\pm$ S.D. = 56% $\pm$ 43%, $P < 0.004$, $n = 8$). The W/S ratio of MAL-6 labeled cytoskeletal proteins increased to approx. 200% control after 10 s exposure to TPCK-trypsin at 0°C, and showed no further increase at 20 s, suggesting that most of the ankyrin was depleted from the membrane between 5 and 10 s.

This suggestion was confirmed by SDS-PAGE. Inspection of Coomassie blue stained SDS-PAGE gels revealed some loss of the ankyrin band at 5 s (Fig. 4, lane C), with almost total loss of band 2.1 after 20 s trypsin treatment (Fig. 4, lane D). Loss of the poorly resolved 2.1 band was accompanied by the loss of 'syndein' bands in the 2.2–2.3 region (Fig. 4). In some cases new, lightly stained bands appeared lower in the gel, just above band 3 in the trypsinized samples (not shown). There was no apparent loss of major bands, except 2.1, at any of the time intervals tested in this experiment. These observations agree closely with those of Cherry and co-workers [20].

Tempamine labeled glycophorin

Previous EPR studies of the specific disruption of the band 4.1-glycophorin linkage by hemin suggested that the physical state of cell-surface sialic acid was altered [15]. We wondered if breakage of the linkage between band 2.1 and a fraction of the band 3 molecules would affect sialic acid motion as well. The motion of the spin label covalently bound to glycophorin was characterized from the spectra like those previously published from our laboratory [23] by an apparent correlation time, τ, which can be thought of as the time required to complete rotation through one radian. The τ parameter was calculated from the well-established equation:

$$\tau = 6.5 \cdot 10^{-10} W_0 \left(\sqrt{\left(\frac{A(0)}{A(+1)} \right)} + \sqrt{\left(\frac{A(0)}{A(-1)} \right)} - 2 \right)$$

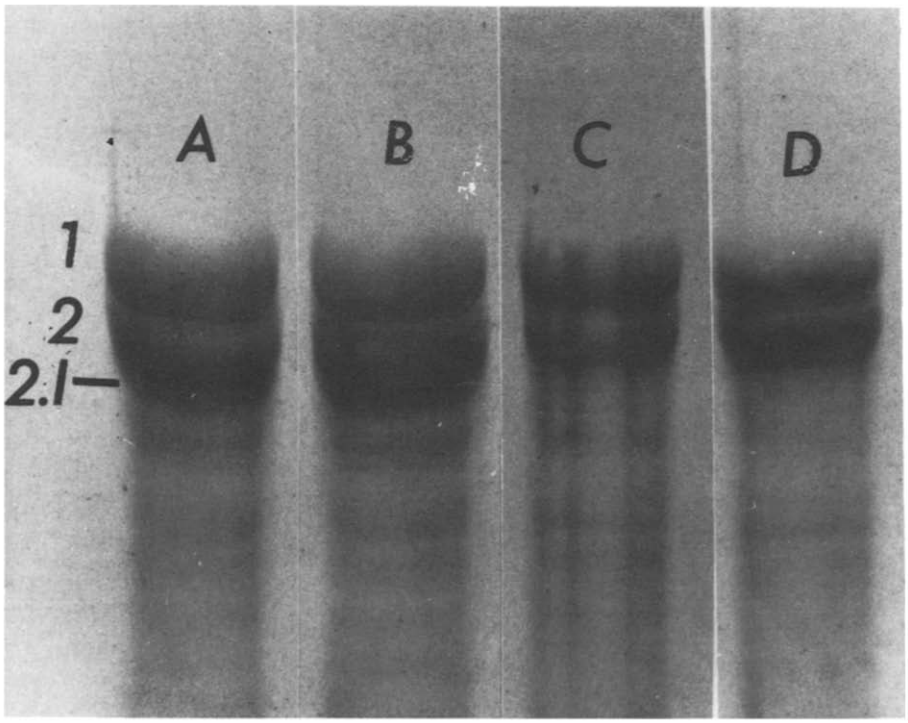

Fig. 4. SDS-PAGE profile of high-molecular weight cytoskeletal components α spectrin (1), β spectrin (2) and ankyrin (band 2.1). Lanes A, B: controls; lane C: 5 s incubation with TPCK-trypsin; lane D: 20 s incubation with TPCK-trypsin as described in text.

where W_0 is the peak-to-peak linewidth of the $M_I = 0$ central line and $A(n)$ refers to the peak-to-peak amplitude of the $M_I = +1$, 0, or -1 lines.

The τ parameter of tempamine bound to sialic acid on glycophorin in ghosts subjected to 5 s trypsin incubation did not differ significantly from controls (mean% control ± S.D. = 97.9 ± 7.01, $P > 0.622$, $n = 4$, Table I). Furthermore, there were no significant effects observed even after 20 s incubation with the proteinase (mean% control ± S.D. = 106 ± 12.3, $n = 4$, Table I). These results suggest that sialic acid motion on glycophorin is unaffected by disruption of the band 3-spectrin linkage upon proteolysis of ankyrin.

Tempamine labeled terminal galactose residues

Since essentially all of the terminal galactose residues specifically spin labeled by reductive amination are located on band 3, band 4.5, and glycophorin [24], and the above-mentioned results suggested that sialic acid on glycophorin was unaffected by trypsin treatment, we reasoned that trypsin cleavage of ankyrin on the cytoplasmic side of a fraction of these transmembrane proteins may affect the motion of terminal galactose residues on the opposite side of band 3 and band 4.5. Incubation of ghosts with TPCK-trypsin for 5–10 s was found to cause a significant decrease in τ relative to controls (mean% control ± S.D. = 90.4 ± 9.29, $P < 0.005$, $n = 12$; Table I), suggesting increased motion and an altered physical state of galactose in cell-surface glycoconjugates after rupture of the band 3-spectrin linkage. As noted above, SDS-PAGE analysis showed no damage to band 3 or band 4.5 as a result of the trypsin treatment described here.

Spermine effect

Previous studies from our laboratory suggested that spermine addition to ghosts electrostatically cross-links negatively charged spectrin to the negatively charged cytoplasmic pole of band 3, thereby increasing protein–protein interactions [28]. We wondered if spermine would 'repair' the connection between band 3 and spectrin caused by trypsin cleavage of ankyrin.

TABLE I

Effect of TPCK-trypsin treatment on τ of tempamine covalently bound to cell-surface carbohydrates

P-values calculated by two-tailed Student's *t*- test. n.s. = not significant; n.c. = not calculated.

Carbohydrate spin labeled	τ (% control) mean ± S.D.	n	P
Sialic acid			
control	100 ± 0.0	4	–
5 s r×n	97.9 ± 7.0	4	n.s.
20 s r×n	105.9 ± 12.3	4	n.s.
Galactose			
control	100 ± 0.0	12	–
5–10 s r×n	90.4 ± 9.3	12	0.005
20 s r×n	88.1 ± 3.2	2	n.c.

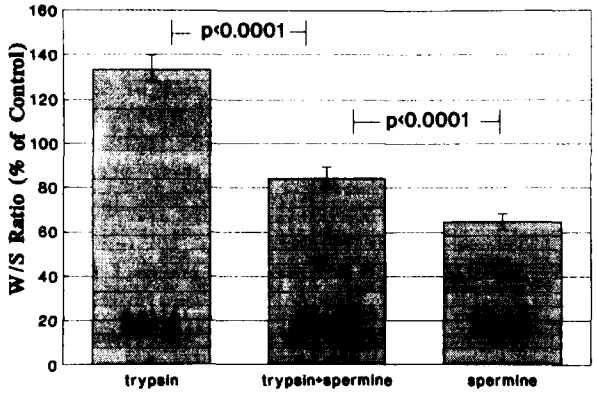

Fig. 5. Effect of spermine addition to untreated ghosts and to ghosts previously treated with TPCK-trypsin. *P* values calculated by one-tailed Student's *t*-test. Error bars indicate S.E.

Fig. 5 shows that the W/S ratio of MAL-6 bound to spectrin was significantly reduced after spermine addition to ghosts previously treated with TPCK-trypsin ($P < 0.0001$), suggesting that spermine partially restored cytoskeletal protein–protein interactions as judged by EPR. The W/S ratio of MAL-6 obtained in this proteinase plus spermine system was some 20% greater than that obtained by addition of spermine to unproteolyzed control membranes ($P < 0.0001$, Fig. 5). Spermine addition to galactose-labeled ghosts previously treated with trypsin showed no effect [τ% control with trypsin treatment, 82.4% ($n = 2$); τ% control with spermine addition after trypsin treatment, 84.2% ($n = 2$)]. This result, coupled with the MAL-6 studies, suggests that the primary effect of this polyamine is to alter the conformation of spectrin upon crosslinking to band 3.

Discussion

The results of our proteolysis experiments closely resemble those of Clague et al. [20], who succeeded in depleting ghosts of ankyrin with a 370:1 (w/w) membrane protein/trypsin ratio and 20 s trypsin incubation. However, other researchers have reported much slower kinetics for this reaction [13]. Although we have no explanation for the results in this latter study, we have noted that the magnitude of the observed W/S increase of the spin label attached to cytoskeletal proteins following trypsin treatment depends somewhat upon the age and batch of proteinase used.

It is not surprising that degradation of ankyrin has no observable effect on the motion of the tempamine spin label bound to the external oligosaccharides of glycophorin: this sialoglycoprotein is not bound directly to ankyrin, and there is probably no permanent bond

between glycophorin and the anion transporter (though some transient band 3-glycophorin interaction is reported [6]).

The trypsin-induced decrease in τ of tempamine bound to terminal galactose residues, which indicates increased motional freedom of the spin label bound to band 3, band 4.5, and glycophorin [24], is extremely interesting. The results with sialic acid labeling may suggest that glycophorin has little role in the effect of trypsin treatment on the motion of terminal galactose. However, since approximately 1/6 of all band 3 molecules in the RBC membrane are thought to bind ankyrin, the remaining 5/6 are presumably free and unbound [1]. Both types of band 3 molecules are labeled by tempamine, so any change in the EPR signal on band 3 resulting from ankyrin degradation arises from only 16% of the total band 3 population. The 10% decrease in τ upon trypsin reaction may therefore represent a drastic physical change in the smaller population of band 3 molecules that has been uncoupled. We can not exclude effects due to band 4.5, although the evidence that this transmembrane protein is coupled to the cytoskeleton is scarce [1].

Knowledge of ankyrin's influence on the physical state of band 3 remains incomplete. Removal of ankyrin (via trypsin) has no effect on band 3 rotational mobility as measured by transient dichroism methods [20]. More recent transient dichroism studies of band 3-labeled erythrocytes suggest that band 3 rotational mobility is restricted only when both ankyrin and band 4.1 are present in the cytoskeleton, which implies synergistic interactions between the two linkage proteins [21]. Though the present study reports no change in the segmental motion of a sialic acid-bound probe upon ankyrin destruction, previous EPR experiments designed to selectively disrupt the glycophorin-band 4.1 linkage (via hemin treatment) suggest diminished segmental motion of galactose-bound tempamine [15]. These various studies indicate that both ankyrin and band 4.1 play complex roles in the molecular motion of band 3 carbohydrates as well as the rotational motion of band 3 itself.

Proteolysis of cytoskeletal elements may be an important step in cell aging processes [2,3,7]. For example, proteolytic removal of a 40 kDa cytoplasmic domain of band 3 has been correlated with IgG autoantibody binding to senescent cells [3,7]. Though speculative at this point, changes in the physical state of cell-surface carbohydrates as a result of proteolysis, or other uncoupling processes, could serve as a signal of aging, leading to several additional biochemical and immunological steps that result in the ultimate removal of the cell.

Spermine is positively charged at pH 8, and, as noted above, has been shown to electrostatically crosslink negatively charged domains of spectrin with the negatively charged cytoplasmic pole of band 3 [28]. The crosslinking effect is evident in Triton X-100 extracted shells, which, in the presence of spermine, retain abnormally high levels of bands 3 and other proteins [28]. Endogenous polyamines may serve a protective or regenerative function [35–37]. Tissue levels of spermine and related polyamines rise in response to mechanical or thermal injuries [36] or ischemia [37]. Correlations have been noted between brain polyamine levels and enhanced proteolysis of high molecular weight proteins [39]. Abnormal cytoskeletal proteolysis, in turn, has been tentatively linked to neuronal degeneration and ischemic injury [40–42]. The role of polyamines in maintenance of cellular homeostasis remains unclear, but our data presented here suggest that these compounds may augment the mechanical function of normal cytoskeletal linkage molecules. We speculate that spermine may act to partially counteract the cytoskeletal perturbation caused by trypsin-mediated proteolysis.

These studies with spin-labeled galactose and previous EPR studies [9,15,28,43,44], suggesting that alterations in specific protein–protein interactions on one side of the membrane can result in alterations in the physical state of membrane components on the opposite side of the membrane, are consistent with a cardinal hypothesis of transmembrane signaling mechanisms, and demonstrate the utility of EPR to investigate membrane structure and functions.

Aknowledgement

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